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### Hansenula polymorpha as cell factory

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## Chapter 5

### Secretion of active alcohol oxidase by *Hansenula polymorpha*.

Meis van der Heide, Ida J. van der Klei, Marten Veenhuis

#### Abstract

In this study, we report the use of the *Saccharomyces cerevisiae* invertase signal sequence (ISS), to mediate secretion of peroxisomal alcohol oxidase (AO) of *Hansenula polymorpha* in the cultivation medium. AO secretion levels increased with increasing copy numbers of the ISSAOX expression cassette integrated into the genome. Only a portion of the produced AO is secreted, the remaining part was observed in ER-like membranous structures that were strongly proliferated. We show that the secreted AO protein is active and has apparently the normal wild-type molecular mass.

## Introduction

The methylotrophic yeast *Hansenula polymorpha* is capable of utilizing methanol as the sole source for carbon and energy. The key enzymes for methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT), are confined to peroxisomes [1].

Peroxisomes are essential organelles in *H. polymorpha* to support growth on methanol. *H. polymorpha* has been of value for fundamental research on peroxisome biogenesis and selective peroxisome degradation. The pronounced morphological features of both processes and the strong induction rates of the key enzymes of methanol metabolism, renders this yeast an attractive model system for these studies [2].

AO is an abundant protein during growth on methanol and has often been used as a model for protein assembly and activation studies [3]. AO is sorted to peroxisomes via a PTS1 type peroxisomal targeting signal (-ARF), which resides at the extreme C-terminus of the protein [4]. Following import of AO into the peroxisomal matrix the protein obtains its enzymatically active form. The activation of AO includes the non-covalent binding of flavin adenine dinucleotide (FAD) as the co-factor and the oligomerization into a homo-octamer of approximately 600 kDa.

In the last decade, methylotrophic yeast, including *H. polymorpha*, also have gained increased attention as host for the production of valuable, heterologous proteins [5, 6]. In a previous report, we used *H. polymorpha* AO as a model protein to study the secretion of large oligomeric proteins. We observed that, using the leader sequence of *Saccharomyces cerevisiae* mating factor  $\alpha$  (MF $\alpha$ ), AO was efficiently targeted into the secretory pathway. The recombinant AO protein was present solely in a monomeric form and lacked FAD. This FAD-less monomeric AO was not stable but subject to gradual endoplasmic reticulum associated degradation (ERAD) [7]. The inability to bind FAD was possibly due to steric hindrance of the FAD binding fold by the pro-region of the MF $\alpha$  leader sequence.

This prompted us to study the use of an alternative ER targeting signal, namely the *S. cerevisiae* invertase signal sequence (ISS). The ISS consists of only a pre-sequence, which is spliced off upon import into the ER thereby rendering an AO protein, which potentially is able to bind FAD at the N-terminus of the protein. In this report, we show the secretion of active octameric AO using this alternative ER targeting signal.

## Materials and Methods

### *Organisms and growth conditions*

All *Hansenula polymorpha* strains used in this study are derivatives of NCYC495 [8]. NCYC 495 *leu1.1* (WT), WT::P<sub>AOX</sub>ISSAOX with various copy numbers and  $\Delta aox::P_{AOX}ISSAOX$  were grown at 37°C in rich complex media (YPD) containing 1% yeast extract, 2% peptone and 1% glucose, in mineral medium as described [9] or on YNB without amino acids containing 0.67% Yeast Nitrogen Base (Difco). Carbon sources used were 0.5% glucose, 0.5% methanol or 0.1% glycerol/0.5% methanol mixtures; as nitrogen source 0.25% ammonium sulphate was used. Leucine was added to final concentrations of 30  $\mu$ g/ml. *Escherichia coli* DH5 $\alpha$

(Gibco-Brl, Gaithersburg, Md) was grown at 37°C in LB medium, supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) when required.

#### *Miscellaneous DNA techniques*

Standard recombinant DNA techniques, *E. coli* transformation and plasmid isolation procedures were performed essentially as described [10]. *H. polymorpha* was transformed by electroporation [11].

#### *Construction of plasmids containing H. polymorpha AO fused to the Invertase Signal Sequence*

The plasmid containing the *S. cerevisiae* Invertase Signal Sequence (ISS) fused in frame to the *H. polymorpha* *AOX* gene under control of the alcohol oxidase promoter ( $P_{AOX}$ ) was constructed as follows: Firstly a plasmid containing the ISS fused to *Aspergillus niger* glucose oxidase was partially digested with *Nhe*I, the isolated 8.9 kb fragment was then digested with *Sma*I resulting in a 7.1 kb vector fragment. The *AOX* gene was amplified using PCR and the *AOX*<sub>start</sub> primer (5' -CCC GGA TCC GCC ATT CCT GAC GAA TTC G) and the *AOX*<sub>stop</sub> primer (5' -CC CCC GGG TTA GAA TCT GGC AAG TCC GGT CTC C). The amplified *AOX* fragment was digested with *Nhe*I and *Sma*I and cloned into *Nhe*I/*Sma*I digested vector fragment. The plasmid pHIPX4-ISS*AOX* was used to transform NCYC495 *leu 1.1* cells. Correct integration into the  $P_{AOX}$  locus was confirmed by Southern blot analysis (data not shown). Strains with various copies of the expression cassette integrated were selected for further analysis.

The fragment used to replace the endogenous *AOX* gene was constructed as follows: a 3.9 kb *Stu*I/*Nar*I fragment from pHIPX4-ISS*AOX* was cloned into pHIPX1 [12] digested with the same enzymes. The resulting plasmid was digested with *Bam*HI and the obtained 8.6 kb fragment was used to transform NCYC 495 *leu1.1* cells. Correct integration into the  $P_{AOX}$  locus and replacement of the chromosomal *AOX* gene was confirmed by Southern blot analysis (data not shown). The resulting strain was designated as  $\Delta aox::P_{AOX}$ ISS*AOX*.

#### *Biochemical methods*

Total cell extracts were prepared as described previously [13]. Deglycosylation using Endo-H (Roche) was performed as described before (Chapter 4 of this thesis).

The presence of FAD in recombinant AO was determined in immunoprecipitates by fluorescence spectroscopy after extraction of FAD from the protein with TCA [14].

SDS-PAGE, Western blotting and determination of AO activity was performed by established procedures. Protein concentrations were determined using the Biorad Protein Assay (Biorad GmbH, Munich, Germany) using BSA as a standard.

#### *Electron microscopy*

Whole cells were fixed and embedded in Epon 812 or unicryl as described previously [15]. Ultrathin unicryl sections were labelled using specific polyclonal antibodies against AO protein, raised in rabbit and goat-anti-rabbit antibodies conjugated to gold according to the instructions of the manufacturer (Amersham, UK).

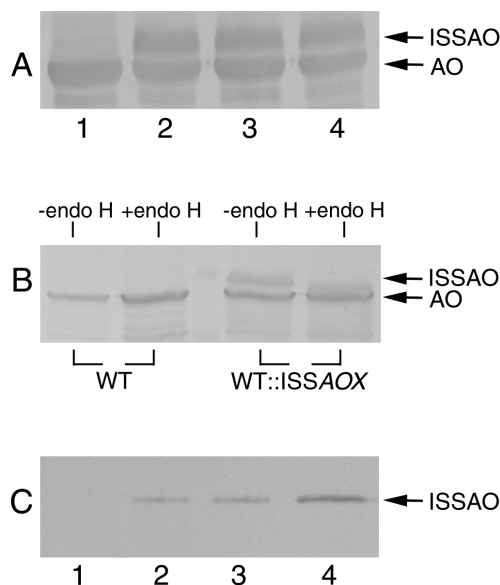
## Results

### *Construction and analysis of strains producing the ISSAO fusion protein.*

In a previous study, we showed that the leader sequence of the *Saccharomyces cerevisiae* mating factor  $\alpha$  (MF $\alpha$ ), efficiently sorted peroxisomal alcohol oxidase (AO) to the secretory pathway, although secretion was prevented. The recombinant AO was found to be monomeric and lacked FAD. We speculated that the inability to bind FAD was due to steric hindrance of the FAD binding fold by the pro-region of the leader sequence as the FAD binding site is near the N-terminus of the protein [16]. To test this hypothesis we used an alternative sorting signal namely the *S. cerevisiae* invertase signal sequence (ISS). This signal differs from that of the MF $\alpha$  in that it consists only of a pre-sequence, which is spliced off upon import of the protein in the ER. As a result, the N-terminus of AO may be normally exposed under these conditions.

To obtain strains in which AO was sorted to the ER using the ISS, a hybrid gene was constructed encoding the ISS fused in frame to *H. polymorpha* AOX gene. This gene was placed under control of the inducible AOX promoter (P<sub>AOX</sub>) and integrated in various copies into the genome of WT *H. polymorpha*.

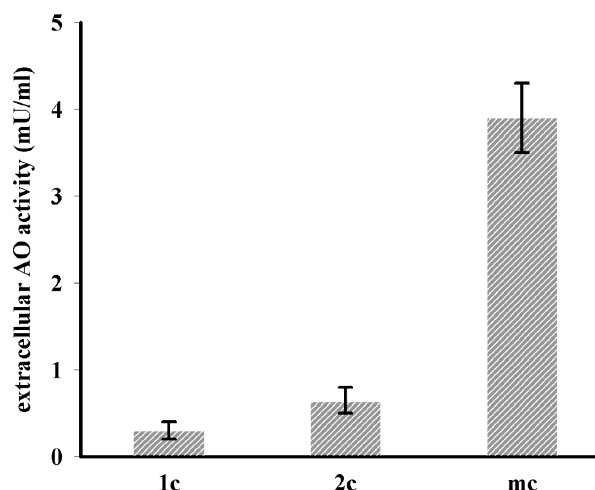
The resulting strains grew on glucose at rates identical to the WT host strain (data not shown). In Western blots, prepared from crude extracts of methanol-grown cells of strains with different copy numbers, decorated with  $\alpha$ -AO antibodies, two AO protein bands were observed (Fig. 1A). The lower band apparently corresponds to normal WT AO protein. The higher band most likely reflects glycosylated AO protein as judged from a deglycosylation experiment, using endoglycosidase H (Endo H) of strain WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> (Fig. 1B). Remarkably, AO protein was also detected in the culture fluid of the various constructed strains (Fig. 1C). From this figure, it is evident that AO protein levels in the culture fluid increased with increasing copy number (Fig. 1C).



**Fig. 1** (A) Western blot prepared from cell extracts of methanol-grown wild type (WT) (lane 1), WT::P<sub>AOX</sub>ISSAOX<sup>1c</sup> (lane 2), WT::P<sub>AOX</sub>ISSAOX<sup>2c</sup> (lane 3) and WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> (lane 4) cells, decorated with  $\alpha$ -AO antibodies. (B) Western blot prepared from samples after deglycosylation using endoglycosidase H (endo H), decorated with  $\alpha$ -AO antibodies. Lanes 1 WT without endo H, 2 WT with endo H, 3 WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> without endo H, 4: WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> with endo H. (C) Western blot prepared from the culture fluids of methanol-grown cells of WT (lane 1), WT::P<sub>AOX</sub>ISSAOX<sup>1c</sup> (lane 2), WT::P<sub>AOX</sub>ISSAOX<sup>2c</sup> (lane 3) and WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> (lane 4). In cells of the recombinant strains two AO protein bands can be observed, representing WT AO (lower band) and the recombinant AO protein which, based on the endo H treatment, is glycosylated (B) compare lanes 3 and 4). Extracellular AO protein

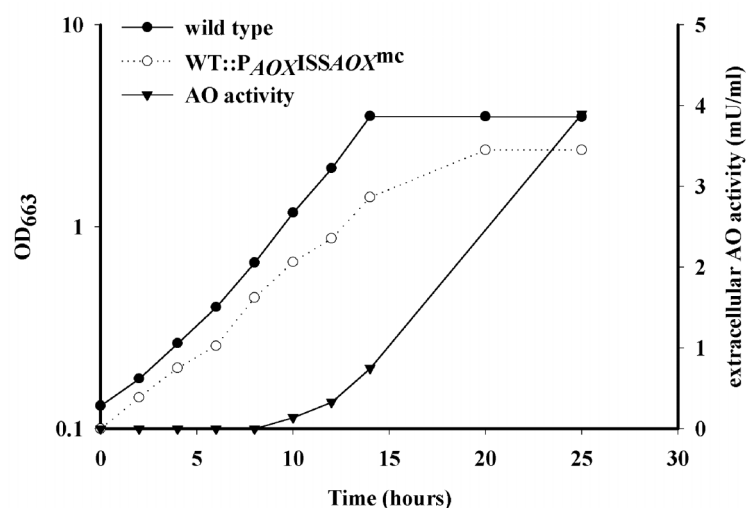
was detected in all recombinant strains (C). For cell extracts, equal amounts of protein were loaded per lane (corresponding to 0.3 OD<sub>660</sub> units of the culture). For the cultivation medium, a volume of the culture medium corresponding to 3 OD<sub>660</sub> units was loaded per lane.

Based on electrophoretic behaviour, the AO protein detected in the culture fluid was of wild type molecular mass and not glycosylated. Activity measurements revealed that the extracellular protein was enzymatically active (Fig. 2). In addition, the increase in AO protein was paralleled by an increase in extracellular AO activity (Fig. 2).



**Fig. 2** Extracellular AO activities after 24 hours of growth on methanol containing medium of strains WT::P<sub>AOX</sub>ISSAOX<sup>1c</sup> (1c), WT::P<sub>AOX</sub>ISSAOX<sup>2c</sup> (2c) and WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> (mc).

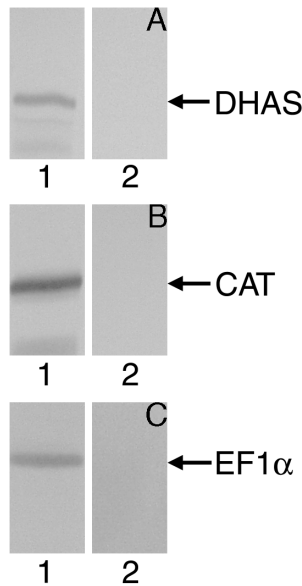
To rule out the possibility that the extracellular AO is due to cell lysis, the WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> strain was grown on methanol and the presence of AO activity in the culture fluid was followed with time (Fig. 3). The data show that extracellular AO appears already in the mid-exponential growth phase, which makes lysis as the cause of extracellular AO unlikely.



**Fig. 3** Growth curves of methanol-grown WT and strain WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> together with extracellular AO activities secreted by WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> in time. During exponential growth, AO activity already can be detected in the culture fluid.

Other evidence arguing against possible cell lysis, was that two other major components of the peroxisomal matrix, DHAS and CAT, were invariable absent in the culture fluid (Fig. 4 A and B). Furthermore, two cytosolic proteins were tested for their presence in both cell extracts and culture fluids of the various grown strains.

Both elongation factor 1 $\alpha$  (EF1 $\alpha$ ) and formate dehydrogenase (FDH), were only detected intracellularly, judged from Western blots decorated with specific antibodies against EF1 $\alpha$  (Fig. 4C) and FDH activity measurements (Table 1).



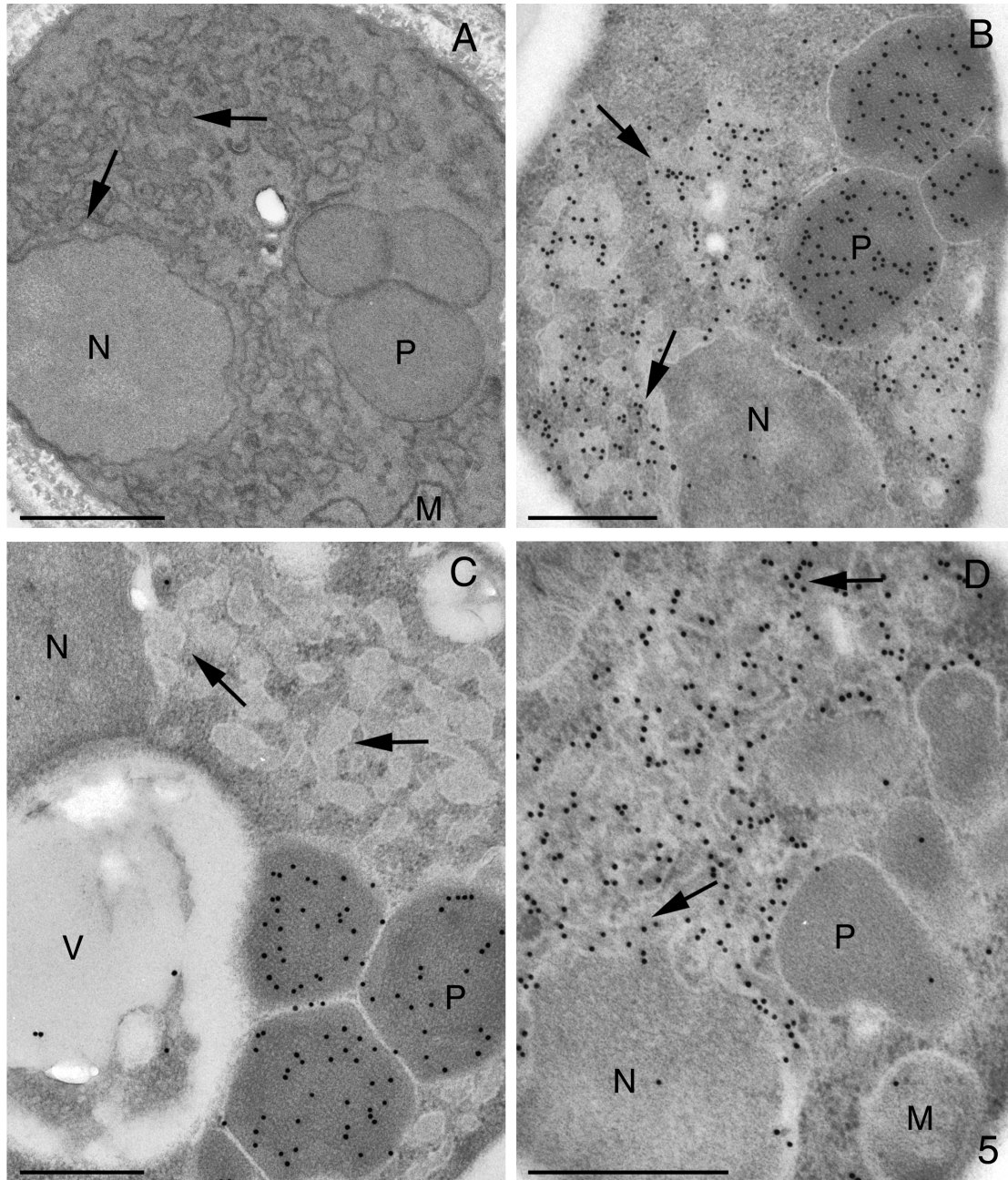
**Fig. 4** Western blots prepared from a cell extract and the culture fluid of methanol-grown WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup>. Blots were decorated using  $\alpha$ -DHAS antibodies (panel **A**),  $\alpha$ -CAT antibodies (panel **B**) and  $\alpha$ -EF1 $\alpha$  antibodies (panel **C**). In all three panels lane 1 represents the crude cell extracts and lane 2 represents the culture fluid. It is clear that none of the tested proteins can be detected in the growth medium. For cell extract, equal amounts of protein were loaded per lane (corresponding to 0.3 OD<sub>660</sub> units of the culture). For the cultivation medium, a volume of the culture medium corresponding to 3 OD<sub>660</sub> units was loaded per lane.

**Table 1** Formate dehydrogenase (FDH) activities determined in crude extracts of the various grown strains.

Strain and Growth condition	FDH activity (U/mg)
WT - Methanol	0.18
WT::P <sub>AOX</sub> ISSAOX <sup>mc</sup> - Methanol	0.19

To ensure that the extracellular AO was indeed originating from the ISSAO fusion, the secreted AO protein was N-terminally sequenced. Here, we made use of the fact that removal of the ISS upon import of the recombinant AO into the ER would result in a serine residue as the first amino acid. Sequencing of the extracellular AO indeed revealed that the first amino acid residue was a serine, thus confirming the recombinant origin of the extracellular AO protein.

The amount of AO protein detected in the culture fluid was not high relative to the total amount of AO produced (Fig. 1 compare A and C). This suggests that bulk of the AO protein had accumulated intracellularly. Indeed, immunocytochemistry, using  $\alpha$ -AO antibodies, on WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> cells revealed that AO protein was present not only on the peroxisomal profiles but also on strongly proliferated ER-like structures (Fig. 5A and 5B). DHAS protein, however, was only present in peroxisomes indicating that proper sorting of DHAS was unaffected in such cells (Fig. 5C).



**Fig. 5** Overall morphology (A) of methanol-grown WT::P<sub>AOX</sub>ISSAOX<sup>mc</sup> cells, showing the presence of the strong proliferated ER-like structures. B shows the results of immunocytochemical experiments using specific antibodies against AO and protein A/gold. Labelling is located on peroxisomes and the ER-like structures. DHAS labelling was exclusively located on peroxisomal profiles, indicating that DHAS sorting was unaffected under these conditions (C). D Ultrathin section of glutaraldehyde-fixed, glycerol/methanol-grown  $\Delta aox::P_{AOX}$ ISSAOX cells labelled with  $\alpha$ -AO antibodies and protein A/gold. AO labelling of strain  $\Delta aox::P_{AOX}$ ISSAOX shows the exclusive presence of AO in the strongly proliferated ER-like structures. N nucleus, M mitochondrion, P peroxisome, V vacuole, arrows ER-like structures. Bar = 0.5  $\mu$ m

#### *Construction and analysis of a $\Delta aox$ strain producing the ISSAO fusion protein*

In order to obtain an *H. polymorpha*  $\Delta aox$  strain, producing the ISSAO fusion protein, a 8.6 kb *Bam*HI fragment was integrated via a double cross-over, into the genome of WT *H. polymorpha*. The resulting strain, designated as



$\Delta aox::P_{AOX}ISSAOX$ , was not able to grow on methanol. However, high  $P_{AOX}$  induction can be achieved upon growth of such cells in batch cultures on glycerol/methanol mixtures. In crude cell extracts, prepared from glycerol/methanol-grown  $\Delta aox::P_{AOX}ISSAOX$  cells, no AO activity was detected. From immunocytochemistry, it was evident that as in  $WT::P_{AOX}ISSAOX$  cells, also in these cells a strong proliferation of ER-like structures had occurred in these cells (Fig. 5D). These structures represented the sole sites of AO protein in the cells. Peroxisome biogenesis, however, was not affected, except that the peroxisomes were smaller of size compared to WT peroxisomes. The lack of AO in these organelles readily explains this observation. Fluorescence analysis of the FAD content of recombinant AO, immunoprecipitated from cell extracts of  $\Delta aox::P_{AOX}ISSAOX$ , revealed that FAD was present in such precipitates. From this, we concluded that the ISSAO fusion protein is able to bind FAD.

## Discussion

In this paper, we show the secretion of active *Hansenula polymorpha* AO, using the leader sequence of the *Saccharomyces cerevisiae* invertase (ISS). Normally, in methanol-grown *H. polymorpha* WT cells, AO is located in peroxisomes. Active AO is an homo-octamer, consisting of eight identical subunits, each of which carries a non-covalently bound FAD as the co-factor.

In a previous study we showed that AO is efficiently targeted to the secretory pathway, using the leader sequence of the *S. cerevisiae* mating factor  $\alpha$  (MF $\alpha$ ). Secretion of AO, however, could not be established under these conditions. Instead, the hybrid AO remained in ER-like structures as a FAD-less monomer. We speculated that the pro-region of the MF $\alpha$  is not cleaved off because the hybrid protein did not reach the site where this cleaving normally occurs, namely in the Golgi. The presence of this pro-region could interfere with the correct exposure of the FAD binding site, which is located at the N-terminus of the protein, thus resulting in a protein which lacks FAD and consequently is unable to assemble properly [17].

In an attempt to overcome these problems, we used the *S. cerevisiae* invertase signal sequence (ISS). A hybrid protein was constructed using 19 amino acids of the ISS fused to full length AO protein. The results showed that AO is efficiently targeted to ER-like structures using the *S. cerevisiae* ISS despite the fact that the peroxisomal targeting signal (-ARF) is still present at the carboxy terminus of the protein. Upon production of the ISSAO fusion protein, a strong proliferation of ER-like structures was observed. Peroxisome biogenesis however, was virtually unaffected under these conditions.

In addition, active AO protein could be detected in the culture fluid of methanol-grown  $WT::P_{AOX}ISSAOX$  cells. The presence of extracellular AO was not due to cell lysis, demonstrated by the fact that both DHAS and CAT were not detectable in the growth medium. Furthermore, EF1 $\alpha$  and FDH activity, both cytosolic proteins, were also invariably absent in the culture fluid under these conditions. The extracellular AO protein is of recombinant origin as became clear from N-terminal sequence analysis.

Surprisingly, the extracellular AO protein is not glycosylated (Fig. 1B). Analysis of the AO protein sequence revealed that AO contains two putative N-

glycosylation sites (Asp-32 and Asp-155). Based on molecular weight estimation of the produced AO protein we propose that one of these sites is glycosylated *in vivo*.

We speculate that glycosylation of AO is leading to conformational changes, which do not allow export of the glycosylated protein from the cell, but which instead, is retained in ER-like structures. Analysis of the FAD content of AO protein located in the proliferated ER-like structures revealed that the recombinant AO is able to bind FAD.

We hypothesise that glycosylation of the recombinant AO possibly leads to a non-exportable protein conformation. This would explain the observation that the extracellular AO, which is active, is not glycosylated. The non-glycosylated AO is therefore the only fraction able to be exported from the cell. To shed further light on this unexpected phenomenon, experiments in which the glycosylation sites are mutagenized are underway.

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